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19 ABSTRACT (Continue on reverse if necessary and identify by block number) Administration of <i>Bordetella pertussis</i> vaccine intraperitoneally induces resistance to mouse adenovirus infection. Subcellular fractions of <i>B. pertussis</i> are capable of inducing resistance also. Boivin antigen, a trichloroacetic acid-soluble extract of whole cells, is capable of inducing resistance in the dose range of 0.2-2.0 ug when adsorbed to alum. Biochemical analysis of Boivin antigen indicated that the lipopolysaccharide and protein components were not complexed, but were co-extracted. The lipopolysaccharide (endotoxin) moiety was responsible for the antiviral activity and the endotoxin-associated proteins potentiated the antiviral effect.					
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1.0 BACKGROUND. Administration of Bordetella pertussis vaccine (BPV) intraperitoneally renders a mouse resistant to a subsequent lethal dose challenge of mouse adenovirus. A similar activity has been reported when type 1 herpes simplex virus was used as the challenging virus. During the first contract year we established that the antiviral activity of BPV was not associated with a given phase of B. pertussis, but was associated with a variety of phase I and phase IV B. pertussis strains (1, see attached reprint). Several subcellular fractions of B. pertussis were shown to have antiviral activity, i.e., a 1.0 M NaCl extract of whole cells (produced by Dr. R. D. Lession, Connaught Laboratories, Inc., Swiftwater, PA), a cell surface polysaccharide removed from whole cells by shearing in a Waring blender, and lipopolysaccharide (LPS) extracted by the phenol-water method of Westphal. All of these fractions contained endotoxin as detected by the Limulus amoebocyte assay (2).

2.0 IDENTIFICATION OF A BPV COMPONENT WITH ANTIVIRAL ACTIVITY. Drs. Barnet Sultzer, John Craig, and R. Castagna, Downstate Medical Center, SUNY, Brooklyn, NY, reported a marked adjuvancy activity following treatment of mice with endotoxin-associated proteins (EP) derived from B. pertussis (3). EP is derived from Boivin antigen, a trichloroacetic acid soluble LPS-rich extract of whole cells. We extracted Boivin antigen from B. pertussis cells and observed antiviral activity. The EP were co-extracted with LPS, but not complexed with LPS. LPS alone induced antiviral activity, but the presence of EP potentiated the activity tenfold or more. Hereafter, Boivin antigen will be designated as EP-LPS to indicate that the antigen contains both lipopolysaccharide and endotoxin-associated proteins.

EP-LPS was extracted in the following manner. B. pertussis cells, strain 10536, were obtained from Dr. Lance Gordon, Connaught Research Institute, Ontario, Canada. Trichloroacetic acid was added to obtain a final concentration of 2.5 % and incubated at 40°C for 3 h. Cell debris was removed by centrifugation at 4,000 x g for 30 min. The supernatant was decanted and the cells were extracted twice with 2.5 % trichloroacetic acid. The supernatants, bright yellow in color, were pooled and filtered through Whatman No. 40 filter paper to remove floating debris, and dialyzed (molecular weight cutoff 12,000-14,000) against chilled tap water overnight. The dialyzed supernatants were concentrated approximately tenfold by flash evaporation. A slight precipitate developed and was removed by centrifugation at 4,000 x g. EP-LPS was precipitated by addition of ethanol (68% final concentration) and incubation overnight at -18°C. The precipitate was sedimented by centrifugation at 4,000 x g for 30 min and washed with 95% ethanol. The sediment was resuspended in endotoxin-free water and dialyzed against distilled water overnight. The solution was frozen and lyophilized. The resulting light brown crystalline powder (EP-LPS) was stored at -18°C.

The EP-LPS antigen contained 1-10 µg endotoxin per µg (dry weight) as measured by the Limulus amoebocyte assay and 680 µg of protein per µg (dry weight) as measured by the Lowry method. Polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) followed by fixation and staining with PAGE blue 83 revealed seven major polypeptides (62K, 57K, 44K, 39K, 34K, 23.5K, and 18K) and 2 minor polypeptides (90K and 32K). Silver staining of gels by the method of Hitchcock and Brown (4) revealed two species of LPS similar to other *B. pertussis* strains (5).

Treatment of C3H/HeN mice with *B. pertussis*-derived EP-LPS antigen rendered the mice resistant to mouse adenovirus infection (Table 1). A dose of 20 µg induced resistance, whereas, a dose of 2.0 µg was not effective (Table 1).

Table 1. Antiviral Activity Associated with EP-LPS Extracted from *Bordetella pertussis*

<u>Treatment*</u>	<u>Mortality (Deaths/Total)</u>
EP-LPS, 20 µg	3/10
EP-LPS, 2.0 µg	10/10
Water (endotoxin-free)	9/10

* EP-LPS was suspended (1.0 µg/ml) in endotoxin-free water and diluted tenfold in endotoxin-free water. Each preparation (0.5 ml) was injected intraperitoneally seven days prior to intraperitoneal inoculation of mouse adenovirus. Experiments were terminated 21 days after infection.

Previous observations obtained with a subcellular fraction of *B. pertussis* prepared by Dr. Robert Lemon, Connaught Laboratories, (1.0 M NaCl extraction, ammonium sulfate precipitation, and Eaulphogene treatment) indicated that lower doses of a subcellular fraction (12 µg) could effect resistance against a virus challenge (2). Eaulphogene treatment to reduce the LPS content resulted in a hydrophobic, insoluble precipitate; therefore, Dr. Lemon's subcellular fraction was adsorbed to alum for administration to test animals. The slow release of Dr. Lemon's fraction from the alum might have increased the potency of the fraction. We adsorbed EP-LPS antigen to the same alum preparation (Alhydrogel) and administered the complex i.p. seven days before challenge with a lethal dose of virus. An increase in potency of EP-LPS antigen was observed (Table 2). As little as 0.02 µg of the alum-adsorbed preparation induced resistance in a portion of the test population.

Table 2. Antiviral Activity Associated with EP-LPS Extracted from Bordetella pertussis and Adsorbed to Alum

<u>Treatment*</u>	<u>Mortality</u> <u>(Deaths/Total)</u>
Experiment 1:	
EP-LPS (20 ug) + Alum	0/10
EP-LPS (2.0 ug) + Alum	1/10
Alum (control)	9/9
Experiment 2:	
EP-LPS (2.0 ug) + Alum	1/10
EP-LPS (0.2 ug) + Alum	4/10
EP-LPS (0.02 ug) + Alum	6/10
Alum (control)	8/10

*EP-LPS was suspended (1.0 mg/ml) in Alhydrogel, E.M. Sargeant Pulp and Chemical Co., Inc., Hoboken, N.J., and diluted to the appropriate concentration in endotoxin-free water. Each preparation (0.5 ml) was injected intraperitoneally 7 days prior to intraperitoneal inoculation of the mouse adenovirus. Experiments were terminated 21 days after infection.

Dr. Barnet Sultzer separated EP-LPS into EP and LPS using phenol-water precipitation of the proteins. Antiviral activity was retained in the LPS fraction, but not in the EP fraction (Table 3). Twenty micrograms of EP adsorbed to alum induced resistance in only a small portion of the test population; whereas 20 ug of LPS induced resistance in most of the test population. A second experiment with 20 ug of LPS did not demonstrate a great degree of protection; thus a 20 ug dose of LPS may be near the dilution endpoint. Additional testing near the 20 ug dose of LPS is planned to confirm the dilution endpoint of LPS.

Table 3. Antiviral Activity Associated with Endotoxin-associated Proteins (EP) or Lipopolysaccharide (LPS) Derived from Bordetella pertussis EP-LPS and Adsorbed to Alum

<u>Treatment^a</u>	<u>Mortality (Deaths/Total)</u>
Experiment 1:	
EP (20 ug) + Alum	9/10
LPS (20 ug) + Alum	1/10
Alum	8/10
Experiment 2:	
LPS (20 ug) + Alum	9/10
LPS (2.0 ug) + Alum	11/11
LPS (0.2 ug) + Alum	10/10
Alum	10/10

^aEP or LPS was suspended (1.0 mg/ml) in Alhydrogel, E.M. Sargeant Pulp and Chemical Co., Inc., Hoboken, N.J., and diluted to the appropriate concentration in endotoxin-free water. Each preparation (0.5 ml) was injected intraperitoneally 7 days prior to intraperitoneal inoculation of the mouse adenovirus. Experiments were terminated 21 days after infection.

Adjuvancy activity alone did not account for the antiviral activity. Dr. Barnet Sultzer observed that the EP extracted from EP-LPS possessed immunomodulatory activities associated with adjuvancy, i.e., both polyclonal activation and mitogenicity. In addition, we have examined a gliding bacteria adjuvant (GBA) for antiviral activity. GBA, provided by Dr. William R. Usinger, University of California, Berkley, CA, has notable adjuvancy activity both in vitro and in vivo (6). The complex polysaccharide is active in picomolar concentrations, stimulates the production of macrophage-derived hormones, IL-2 colony stimulating factor, and tumor necrotizing factor, and stimulates B cell proliferation and immunoglobulin secretion; however, the polysaccharide failed to induce resistance to mouse adenovirus under our test conditions (Table 4). We plan to test higher concentrations of GBA (20-200 ug) for antiviral activity.

Table 4. Lack of Antiviral Activity Associated with Gliding Bacteria Adjuvant Derived from Cytophaga

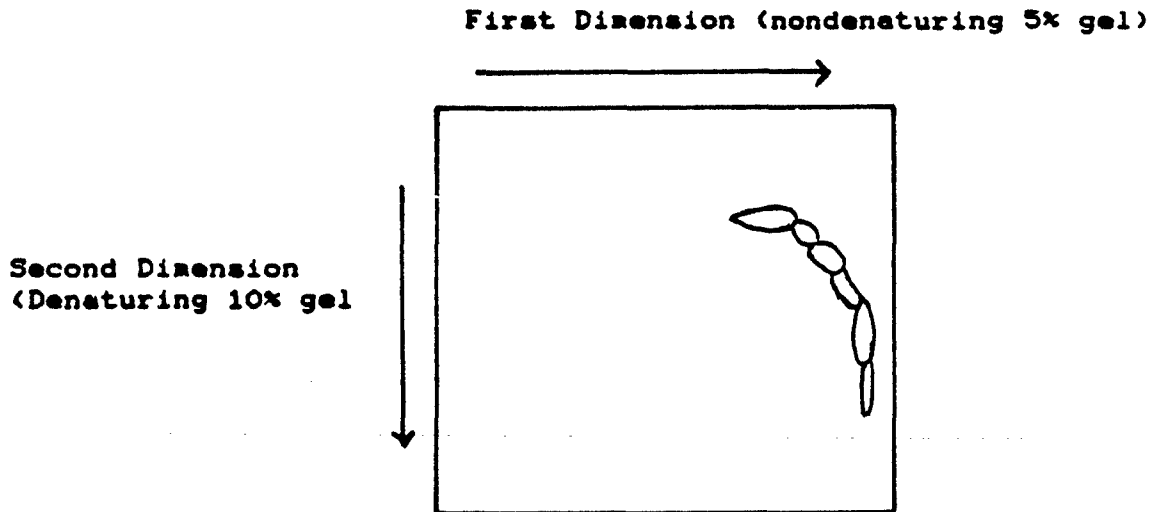
<u>Treatment*</u>	<u>Mortality (Deaths/Total)</u>
GBA (20 ug) + Alum	10/10
GBA (2.0 ug) + Alum	9/9
GBA (0.2 ug) + Alum	10/10
Alum	9/10

* GBA was suspended (1.0 mg/ml) in Alhydrogel, E.M. Sargeant Pulp and Chemical Co., Inc., Hoboken, N.J., and diluted to the appropriate concentration in endotoxin-free water. Each preparation (0.5 ml) was injected intraperitoneally 7 days prior to intraperitoneal inoculation of the mouse adenovirus. Experiments were terminated 21 days after infection.

It appears that the EP in EP-LPS potentiates the antiviral activity of LPS approximately 10- to 100-fold. We are currently examining the activity of both EP and GBA in reconstruction experiments to determine whether the interaction of these adjuvants with B. pertussis-derived LPS will potentiate the antiviral activity.

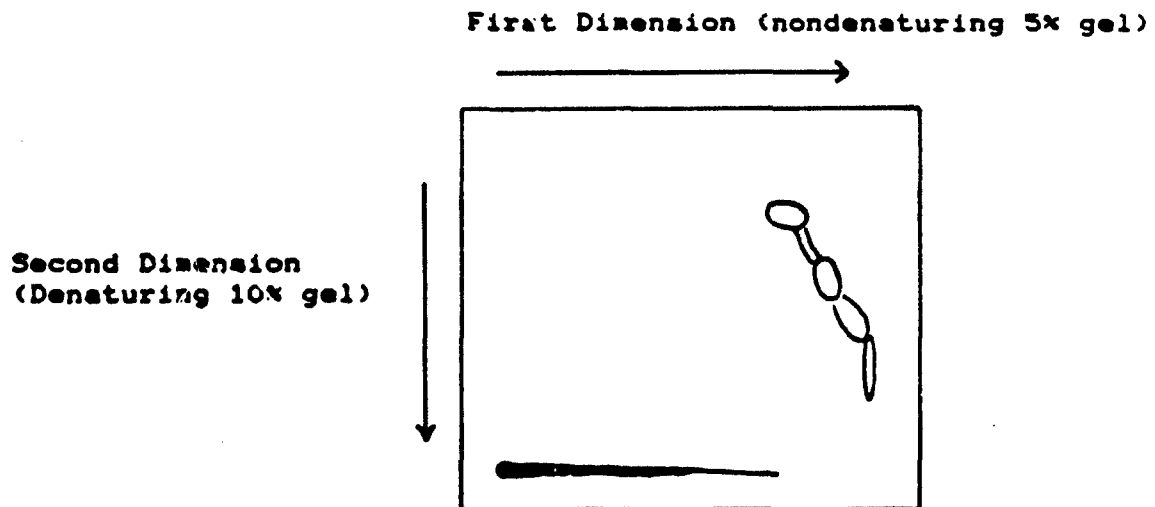
We have examined EP-LPS to determine whether a unique complex of LPS and protein existed. EP-LPS was electrophoresed in a two-dimensional gel. The first dimension consisted of nondenaturing conditions in a cylindrical 5% acrylamide gel and discontinuous buffers (stacking gel, pH 6.8; resolving gel pH 8.8) to separate the proteins of EP-LPS by charge and not by size (7). After a short equilibration of the first dimension cylindrical gel in SDS-buffer (pH 6.8) the proteins were then electrophoresed in a second dimension consisting of denaturing conditions in a slab 10% acrylamide gel and SDS-buffer (pH 8.8) to separate the proteins by molecular size (8). If EP-LPS existed as a complex then the proteins would be detected in a vertical line parallel with the direction of electrophoresis in the second dimension. PAGE Blue 83 staining of the two dimensional gel revealed the proteins of EP-LPS in the righthand side of the gel and most of the proteins were not oriented in a straight line parallel to the direction of electrophoresis in a denaturing SDS gel (Figure 1). Under the conditions used for electrophoresis the proteins of EP-LPS do not appear to be extracted as a complex.

Figure 1. Two Dimensional Gel Electrophoresis of EP-LPS followed by Protein Stain



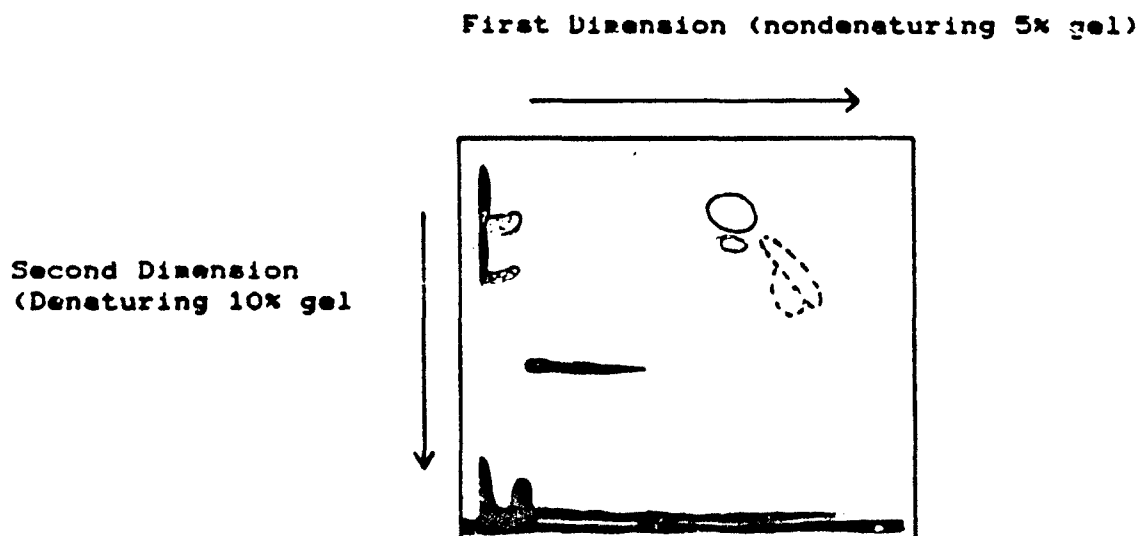
Silver staining of LPS followed by PAGE Blue 83 staining revealed the LPS of EP-LPS in the lower lefthand corner of a two dimensional gel and the LPS was well-separated from the proteins (Figure 2).

Two Dimensional Gel Electrophoresis of EP-LPS followed by Silver Staining and Protein Staining



EP-LPS was separated by two dimensional electrophoresis and transferred to nitrocellulose paper by electrophoretic transfer at a pH of 8.3 (9). The nitrocellulose paper was blocked with 10% nonfat dry milk. The blocked paper was then incubated with rabbit antiserum raised against BPV (Connaught Laboratories, Inc.), washed, and incubated with goat anti-rabbit IgG conjugated with horse radish peroxidase. The immunoblot was developed with 4-chloro-1-naphthol and hydrogen peroxide (10). LPS was the immunodominant antigen in EP-LPS. As observed with the silver stain, the two species of B. pertussis LPS was observed in the lower lefthand corner of the two dimensional gel and well separated from the proteins (Figure 3). A single immunodominant protein may have been complexed with LPS during the extraction. This protein migrate poorly in the first dimension and was marginally visible on the PAGE Blue 83 stained two dimensional gels. Taken together, these data indicate that EP-LPS is probably not extracted as a complex, but the components are co-extracted in the 2.5% trichloroacetic acid. Additional two dimensional gels will be silver stained for protein to ascertain the number and characteristics of minor proteins in the EP-LPS extract. We also will examine the purified LPS by Western blot analysis to determine whether the single immunodominant protein observed in EP-LPS was removed by phenol-water precipitation.

Figure 3. Two Dimensional Gel Electrophoresis of EP-LPS followed by Electrophoretic Transfer To Nitrocellulose Paper and Staining with Enzyme-linked Antibody to BPV (Western Blot).



LPS, derived from strains 3779 BL2S₄ (2) and 10536 and purified by two different methods, elicits antiviral activity in C3H/HeN mice. These observations support a hypothesis that LPS plays an important, if not a singular role, in the antiviral activity associated with B. pertussis. Using purified LPS we can now begin to examine in detail the immunomodulatory mechanisms that develop following administration of LPS and hopefully associate given mechanisms with the virus resistant state. In addition we can begin to assess the role of adjuvants in this phenomenon.

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Resistance to Adenovirus Infection After Administration of *Bordetella pertussis* Vaccine in Mice

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Treatment of mice with *Bordetella pertussis* vaccine rendered mice resistant to mouse adenovirus infection. The resistant state took at least 5 days to develop, and susceptibility returned to a portion of the test population 35 days after treatment. Transient resistance developed in congenitally athymic mice also. Treatment with a dose of 25 µg (dry weight) of *B. pertussis* vaccine protected approximately 50% of the test population. Vaccines prepared from several different strains of *B. pertussis* were capable of inducing resistance, and the induction of resistance was not dependent on the mouse strain used for testing. Cross-reacting antibodies capable of neutralizing the virus or protecting against a challenging infection were not induced by treatment with *B. pertussis* vaccine.

A variety of immunomodulatory activities have been associated with *Bordetella pertussis* vaccine (BPV) (14, 17, 19, 20). Acellular components extracted from the *B. pertussis* cell can mimic some of these immunomodulatory activities (3, 4, 7, 8, 18, 22, 23). Treatment of mice with BPV or components extracted from *B. pertussis* can modify the pathogenesis of virus infections. Increased susceptibility to intranasal influenza virus challenge was observed 5 to 7 days after intraperitoneal (i.p.) injection of BPV (21). Increased resistance to intracranial rabies virus challenge was observed when an extract of *B. pertussis* was administered by the subcutaneous, intravenous, or i.p. route at the same time as the virus (19). Increased resistance to i.p. herpes simplex virus challenge was observed 7 days after i.p. injection of BPV; however, resistance was not observed when BPV was administered 3 days before virus challenge (13). Several acellular fractions of *B. pertussis*, namely, lipopolysaccharide, glycolipid, lipid A, or lipid X, induce a state of resistance to an i.p. challenge of encephalomyocarditis virus or a subcutaneous challenge of Semliki Forest virus when the *B. pertussis*-derived fractions were administered i.p. 24 h previously (1, 3). Discussions with the late Charles W. Fishel concerning the immunomodulatory activities of *B. pertussis* and the reports of antiviral activity of BPV suggested that BPV might modulate mouse adenovirus infection by a plaque-type variant of mouse adenovirus strain FL. The plaque-type variant strain, designated MA1p14, induces an interstitial pneumonia and death (25). Increased resistance to an i.p. challenge of mouse adenovirus was observed when BPV was injected i.p. 7 days before virus challenge (14). This report extends the characterization of BPV-induced resistance to mouse adenovirus infection.

MATERIALS AND METHODS

Animals. Female mice, strain BDF₁/Cox (C57Bl/6 Cox × DBA/2 Cox) were obtained from Laboratory Supply Co., Indianapolis, Ind. A colony of C3H/HeN (mammary tumor virus positive [MTV⁺]) mice with the nude gene mutation

was obtained from Carl Hansen, National Institutes of Health, Bethesda, Md., and bred and maintained at the University of South Florida, Tampa, Fla., as described previously (24). Male mice heterozygous for the nude gene and female mice homozygous for the nude gene were used in this study. A colony of C3H/HeN (MTV⁻) mice was obtained from Carl Hansen and bred and maintained at the University of Alabama, University. Both male and female mice were used in this study.

Vaccines. BPV was provided by Connaught Laboratories, Swiftwater, Pa., and was adjusted to approximately 4.0 mg (dry weight) per ml in saline-thimerosal diluent (0.15 M NaCl in 0.02% thimerosal). Vaccines were made also from *B. pertussis* strains 18323 (James L. Cowell, Food and Drug Administration, Bethesda, Md.) and Tohama I (Stanley Falkow, Stanford University, Stanford, Calif.). The organisms were maintained on BG agar base (Difco Laboratories, Detroit, Mich.) supplemented with 17% defibrinated sheep blood. Vaccines were made by harvesting 4-day growth from Cohen-Wheeler agar in phosphate-buffered saline (pH 7.2) and inactivating the cells by heating (56°C for 30 min) in the presence of 0.02% thimerosal. The vaccines were adjusted to 4.0 mg (dry weight) in saline-thimerosal diluent and stored at 4°C.

Virus. MA1p14 was propagated in L cells (NCTC clone 929; American Type Culture Collection, Rockville, Md.) The virus titer of infecting stocks was determined by a plaque assay (24).

Leukocytosis. Mice were bled from the retroorbital plexus with heparinized capillary tubes. Samples (10 µl) of peripheral blood from each mouse were placed into tubes containing 490 µl of Turk solution (9), mixed, and counted with a hemocytometer.

Electron microscopy. Cells obtained by peritoneal lavage were sedimented by centrifugation at 450 × g for 10 min. The cell pellet was covered with fixative (2% paraformaldehyde-3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.3) and postfixed with osmium tetroxide. After the cell pellet was embedded in epon, ultrathin sections (60 to 70 nm) were stained as previously described (24).

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TABLE 1. Induction of resistance to MAD1p14 challenge by BPV administration

Mouse strain	Treatment ^a	Virus dose (PFU)	Mortality (deaths/total)
C3H/HeN (+/nu) (MTV ⁻)	Diluent	1.0×10^8	25/25
C3H/HeN (+/nu) (MTV ⁻)	BPV (Connaught)	1.0×10^8	0/20
BDF ₁	Diluent	1.0×10^8	20/20
BDF ₁	BPV (Connaught)	1.0×10^8	0/20
C3H/HeN (MTV ⁻)	Diluent	2.2×10^7	21/21
C3H/HeN (MTV ⁻)	BPV (Connaught)	2.2×10^7	0/21
C3H/HeN (MTV ⁻)	Diluent	2.8×10^7	10/10
C3H/HeN (MTV ⁻)	BPV (13323)	2.8×10^7	0/10
C3H/HeN (MTV ⁻)	Diluent	2.8×10^7	5/5
C3H/HeN (MTV ⁻)	BPV (Tohama I)	2.8×10^7	0/5

^a Animals were injected i.p. with either saline-thimerosal diluent or 250 μ g (dry weight) of BPV 7 days before i.p. virus challenge.

RESULTS

Induction of resistance. Treatment of mice with BPV induced resistance to an MAD1p14 challenge (Table 1). Animals were injected i.p. with BPV; 7 days later, lethal doses of MAD1p14 were administered. The animals were observed thereafter for clinical disease and death accompanied by hemorrhagic lungs at necropsy. Treatment of mice with BPV induced a resistant state to as much as 20-50% lethal doses of the virus. The treatment protected the mice also from overt clinical signs of disease, such as hunched back, ruffled fur, marked inactivity, and huddling. The long-term survival of mice after BPV treatment and MAD1p14 infection was at least 65 days. The longest duration of disease and death observed after MAD1p14 infection was 11 days; therefore, experiments were scored 21 days after infection. The 50% effective dose of BPV was approximately 25 μ g (dry weight) or approximately 1.2 mg/kg of body weight (Table 2).

The kinetics of the induction of the resistant state suggest that BPV was not interacting directly with the virus. The resistant state did not develop immediately after administration of the vaccine, but was observed initially 5 days after BPV treatment (Table 3). The resistant state was transient, and susceptibility returned to a portion of the test population 35 days after BPV treatment.

A functioning thymus was not necessary for induction of the resistant state. Congenitally athymic mice were very susceptible to MAD1p14 infection (Table 4). The kinetics of disease and death after high-dose virus infection was similar

TABLE 2. Dose response of BPV-induced resistance to MAD1p14 in BDF₁ mice

Treatment ^a	Mortality (deaths/total)
Diluent	10/10
2.5 μ g of BPV	10/10
12.5 μ g of BPV	10/10
25 μ g of BPV	19/47
125 μ g of BPV	1/10
250 μ g of BPV	0/10

^a Mice were injected i.p. with the designated amounts (dry weight) of BPV (Connaught Laboratories) 7 days before i.p. virus challenge with 1.0×10^8 PFU.

TABLE 3. Kinetics of BPV-induced resistance to MAD1p14

Expt	Mouse strain	Time of treatment ^a (days)	Virus dose (PFU)	Mortality (deaths/total)
1	BDF ₁	0.02	1.0×10^8	5/5
	BDF ₁	7	1.0×10^8	0/10
	BDF ₁	14	1.0×10^8	0/10
	BDF ₁	21	1.0×10^8	0/10
	BDF ₁	35	1.0×10^8	4/10
2	C3H/HeN (MTV ⁻)	1	2.1×10^7	5/5
	C3H/HeN (MTV ⁻)	2	2.1×10^7	5/5
	C3H/HeN (MTV ⁻)	3	2.1×10^7	5/5
	C3H/HeN (MTV ⁻)	4	2.1×10^7	5/5
	C3H/HeN (MTV ⁻)	5	2.1×10^7	0/5
	C3H/HeN (MTV ⁻)	6	2.1×10^7	0/5
	C3H/HeN (MTV ⁻)	7	2.1×10^7	0/5

^a Mice were injected i.p. with 250 μ g (dry weight) of BPV (Connaught Laboratories) and challenged with virus at the designated times of treatment. Both experiments included diluent injected groups of mice as controls; the control groups exhibited 100% mortality.

in both thymus-bearing and athymic animals. Infection with low doses of the virus produced no overt disease or death in thymus-bearing animals, whereas low-dose infection of athymic animals produced a chronic wasting disease and death with hemorrhagic lungs after an extended period of time. A transient BPV-induced resistant state was observed also in congenitally athymic mice. In contrast to the thymus-bearing animals, the athymic animals succumbed to high-dose infection and disease after the resistant state decayed (Table 4).

TABLE 4. Dose response of MAD1p14 in normal, athymic, and BPV-treated athymic mice

Mouse strain	Virus challenge (PFU)	Mortality (deaths/total)	Time of death (days)
BDF ₁	1.0×10^8	10/10	3, 3, 3, 3, 3, 3, 3, 3, 3, 3
BDF ₁	1.0×10^7	10/10	3, 3, 3, 3, 3, 3, 3, 3, 3, 3
BDF ₁	1.0×10^6	2/10	3, 3
C3H/HeN (+/nu)	1.0×10^8	15/15	3, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4
C3H/HeN (+/nu)	1.0×10^7	8/10	6, 8, 8, 8, 8, 10, 10, 10
C3H/HeN (+/nu)	1.0×10^6	1/10	9
C3H/HeN (nu/nu)	1.0×10^8	5/5	5, 5, 5, 5, 6
C3H/HeN (nu/nu)	1.0×10^7	5/5	8, 10, 16, 21, 33
C3H/HeN (nu/nu)	1.0×10^6	5/5	18, 18, 22, 26, 31
C3H/HeN (nu/nu)	1.0×10^5	4/5 ^a	35, 38, 38, 38
C3H/HeN (nu/nu)	1.0×10^4	5/5	26, 30, 33, 35, 39
C3H/HeN (nu/nu)	1.0×10^3	5/5	25, 29, 33, 35, 36
C3H/HeN (nu/nu)	1.0×10^2	5/5	33, 33, 36, 36, 45
BPV-treated C3H/HeN (nu/nu) ^b	1.0×10^8	15/15	7, 11, 37, 38, 39, 40, 43, 43, 46, 46, 48, 51, 54, 54, 63
Diluent-treated C3H/HeN (nu/nu) ^b	1.0×10^8	15/15	4, 4, 4, 4, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5, 5

^a The survivor was sacrificed by cervical dislocation 149 days after infection.

^b Mice were injected with vaccine diluent or 250 μ g (dry weight) of BPV (Connaught Laboratories) 7 days before virus challenge.

TABLE 5. Effect of heat treatment on the antiviral activity of BPV

Mouse strain	Treatment ^a	Mortality (deaths/total)
C3H/HeN (+/nu)	Diluent	15/15
C3H/HeN (+/nu)	BPV	0/10
C3H/HeN (+/nu)	Heated BPV ^b	0/15
BDF ₁	Diluent	5/5
BDF ₁	BPV	0/10
BDF ₁	Heated BPV ^b	5/10

^a Mice were injected i.p. with vaccine diluent or 250 µg (dry weight) of BPV (Connaught Laboratories) 7 days before virus challenge.

^b BPV (Connaught Laboratories) was incubated at 80°C for 30 min before i.p. injection.

Treatment of BPV to inactivate pertussigen (80°C for 30 min) (19) did not affect resistance-inducing activity when assayed in C3H/HeN (+/nu) mice, but decreased activity when assayed in BDF₁ mice (Table 5). The heated vaccine failed to induce lymphocytosis in the peripheral blood of mice (Table 6).

Effects of treatment with BPV. High-dose MAd1p14 infection did not effect a notable generalized immune response (splenomegaly) within 3 days of virus inoculation (Table 7). Treatment of mice with BPV induced a threefold increase in spleen size, and the splenomegaly was retained during 3 days of high-dose virus infection.

High-dose MAd1p14 infection did not induce an observable increase in the number of peritoneal leukocytes 3 days after infection (Table 8). Similar to a previous study of Fishel and coworkers (5), BPV treatment effected a fivefold increase in peritoneal leukocyte number. The combination of BPV treatment and high-dose virus infection had a synergistic effect, and the peritoneal leukocyte number was increased approximately 10-fold. The activity of the BPV-induced peritoneal lavage cells increased markedly. Electron microscopy of diluent-treated peritoneal exudate cells 3 days after high-dose virus infection revealed numerous virus particles in the extracellular medium and in phagolysosomes (Fig. 1A). In contrast, BPV-treated peritoneal exudate 3 days after high-dose virus infection exhibited virtually no cells with virus particles (Fig. 1B).

Antibodies did not play a role in the development of the BPV-induced resistant state. Treatment of mice with BPV for 10 days did not induce MAd1p14 neutralizing antibodies. Studies with monoclonal antibodies indicate that certain classes of antibodies do not neutralize, but will protect the test animal against a challenging virus infection (2, 15, 16). Treatment of mice with BPV for 21 days did not induce protective antibodies. Serum (0.5 ml) from the BPV-treated

TABLE 6. Effect of heat on lymphocytosis after BPV treatment of C3H/HeN (+/nu) mice

Treatment ^a	Peripheral blood leukocytes (cells/mm ³)	
	Mean	SD
Diluent	6,380	260
BPV	17,600	3,800
Heated BPV ^b	7,600	820

^a Mice were injected i.p. with vaccine diluent or 250 µg (dry weight) of BPV (Connaught Laboratories) 4 days before bleeding.

^b BPV (Connaught Laboratories) was incubated at 80°C for 30 min before i.p. injection.

TABLE 7. Effect of BPV treatment and MAd1p14 infection on spleen weight^a

Treatment		Avg spleen wt on day 10 (g)
Day 0	Day 7	
Diluent	Medium	0.090
Diluent	1.0 × 10 ⁸ PFU	0.130
BPV	Medium	0.352
BPV	1.0 × 10 ⁸ PFU	0.338

^a Groups of three C3H/HeN (+/nu) mice were injected i.p. with vaccine diluent or 250 µg (dry weight) of BPV. Seven days later, the mice were injected i.p. with cell culture medium or virus stock. After 3 days the mice were sacrificed by cervical dislocation, and the spleens were removed, blotted to remove excess fluid, and weighed.

C3H/HeN (MTV⁻) mice was injected i.p. into mice of the same strain and challenged with a high-dose virus inoculum 24 h later. Assuming a plasma volume of 1.1 ml (6), then potentially protective antibodies would have been diluted approximately threefold. A group of 10 mice treated in this manner succumbed to high-dose infection within 7 days.

DISCUSSION

Mouse adenovirus can be added to the list of virus infections that are modulated by treatment of the test animal with *B. pertussis* or materials derived from the microorganism. Development of the resistant state was not dependent on the strain of the microorganism. Although the *B. pertussis* strain from Connaught Laboratories, Inc., and strain 18323 (12) have a common lineage, strain Tohama I was derived from a completely different source (10). Development of the resistant state also was independent of the strain of mouse used.

The kinetics of development and decay of the resistant state indicated that a relatively long-term modification of the immunological mechanisms occurred. The modification probably did not involve thymus-derived lymphocytes in its mechanism of development, since congenitally athymic mice responded to BPV treatment with resistance. However, thymus involvement in the BPV-induced resistance in the thymus-bearing animal cannot be ruled out due to possible compensatory immune functions operating in congenitally athymic mice, such as increased numbers of natural killer cells. Thymus-derived cytolytic lymphocytes are necessary for final clearance of certain virus infections (11); consequently, the mechanism of BPV-induced resistance in thy-

TABLE 8. Effect of BPV treatment and MAd1p14 infection on peritoneal leukocytes^a

Treatment		Leukocytes in Peritoneal Lavage (cells × 10 ⁻⁶ per ml)			
		Day 7		Day 10	
Day 0	Day 7	Mean	SD	Mean	SD
Diluent		0.9	0.1	1.0	0.2
BPV		5.2	0.8	5.5	0.5
Diluent	1.0 × 10 ⁸ PFU			1.1	0.2
BPV	1.0 × 10 ⁸ PFU			10.0	1.0

^a Groups of six C3H/HeN (+/nu) mice were treated by i.p. inoculation of vaccine diluent or 250 µg (dry weight) of BPV. Seven days later three mice from each group were sacrificed by cervical dislocation, and 4.0 ml of Dulbecco modified minimal Eagle medium was injected into the peritoneum. The peritoneal wall was massaged, 3.5 ml of lavage was withdrawn, and the cells were counted. The remaining three mice in each group were inoculated with virus and sacrificed for lavage 3 days later.

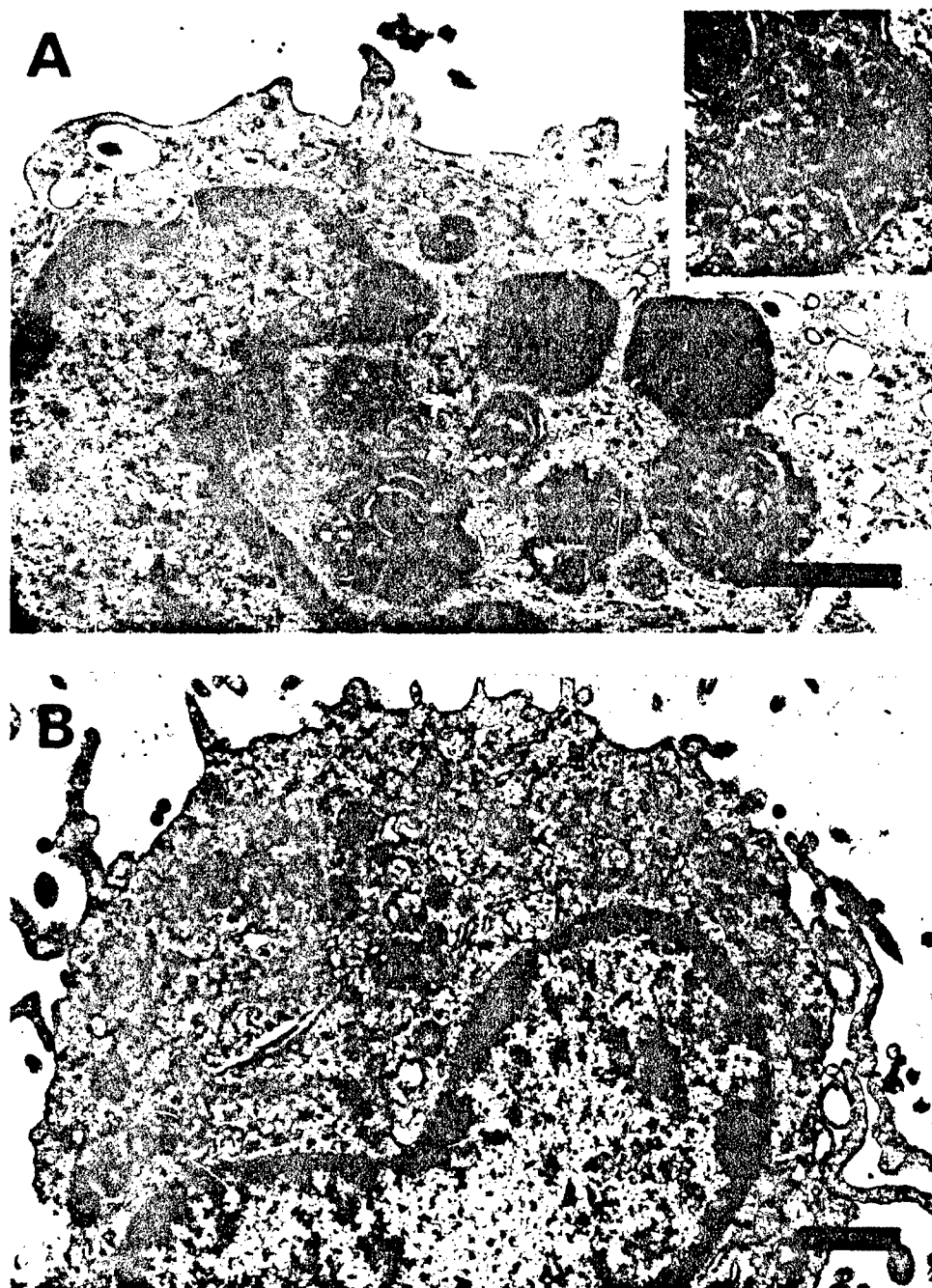


FIG. 1. Electron micrograph of ultrathin sections of peritoneal lavage cells from C3H/HeN (+/nu) mice. Bar, 1.0 μ m. (A) Representative cell obtained after treatment with vaccine diluent (7 days) followed by MAd1p14 infection (3 days). The inset shows an increased magnification of a phagolysosome containing virus particles. (B) Representative cell obtained after BPV treatment (7 days) followed by MAd1p14 infection (3 days).

mus-bearing mice is probably an expression of retarded virus growth until the proper T-lymphocyte clones can develop. This conclusion is supported by the similarity of disease patterns in BPV-treated and low dose-infected athymic mice.

The immune response of mice appears to be overwhelmed by high-dose MAd1p14 infection; death occurs before a systemic response in the form of splenomegaly, leuko-

cytosis, and increase leukocytes in the peritoneum can develop. The treatment of mice with BPV could provide the mouse with a systemic response at the time of virus inoculation; however, one must consider that clinical signs of systemic response might not be involved in the resistant state. We have observed that acellular fractions of *B. pertussis* have decreased ability to induce splenomegaly and leukocytosis, yet retain resistance-inducing activity (R. S.

Stinson, J. D. Lee, L. Williamson, and A. Winters, Abstr. 19th Natl. Meet. Reticuloendothel. Soc. 1982, abstr. no. 56, p. 70). The mechanism of BPV-induced resistance markedly decreased virus particles from the inoculum or subsequent multiplication in peritoneal lavage cells obtained 3 days after infection (or both).

Study of the molecules or complex of molecules in *B. pertussis* that are responsible for induction of the virus-resistant state, and investigation of their mechanism of immunomodulation might lead to new methods for prevention and treatment of virus infections.

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